

ANTINEOPLASTIC AND APOPTOTIC EFFECTS OF CANNABINOIDS. N-ACYLETHANOLAMINES: PROTECTORS OR KILLERS?

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The proapoptotic and antineoplastic properties of cannabinoids with emphasis on effects of *N*-acylethanolamines were analyzed. Cannabinoids enhanced apoptotic and necrotic processes in many types of tumour cells and tissues. Involvement of different types of receptors and signaling pathways in mediating the proapoptotic effects of cannabinoids are discussed. The evidences in favour of both proapoptotic, pronecrotic and protective, antiapoptotic effects of cannabinoids and, especially *N*-acylethanolamines, are evaluated. The hypothesis is suggested that *N*-acylethanolamines, formed in some tissues under strong stress conditions, can be not a consequence of tissue damage but cause such damage. The conclusion is made on promising of cannabinoids as potential anticancer agents.

Key Words: Cannabinoids, *N*-acylethanolamines, apoptosis, necrosis, tumours, ischemia.

In the last years, the endocannabinoid system has emerged as a highly relevant topic in the scientific community [1–9]. Many different regulatory actions have been attributed to cannabinoids in immune, cardiovascular, respiratory, digestive, reproductive, ocular and central nervous systems, and their involvement in several pathophysiological conditions has been shown. They produce a wide spectrum of central and peripheral effects, such as alterations in cognition and memory, immunosuppression, analgesia, anticonvulsion, anti- and proinflammation, and alleviation of intraocular pressure in glaucoma, cachexia, emesis and nausea. CB ligands also have vasorelaxing, cytostatic, apoptotic, and necrotic effects. They may be effective agents in the treatment of pain, glaucoma, neurodegenerative disorders such as Parkinson's disease, multiple sclerosis, and the wasting and emesis associated with AIDS and cancer chemotherapy. In addition, CBs might be potential antitumoral agents. They can modulate apoptosis, cell migration, survival, proliferation, and differentiation.

Endocannabinoid research received a characterization of the chemical structure of THC, the main psychoactive constituent of marijuana. THC was prompted by the discovery of specific brain receptors. The existence of these receptors implies the presence of endogenous ligands, and in the early 1990s the first endocannabinoid, anandamide (AEA), which is the ethanolamide of arachidonic acid. AEA was

shown both to occupy CB receptors and to mimic the functional effects of THC. It now seems that AEA is the prototype of a family of *N*-acylethanolamines that have similar effects via activation of G protein-coupled CB receptors.

1. RECEPTORS

1.1. Cannabinoid receptors

The endocannabinoids as well as plant-derived and synthetic CBs were shown to activate two distinct CB receptors: the CB1 receptor, which is present at high concentrations in the nervous system [1, 10, 11], and the CB2 receptor, which is located primarily in immune cells [1, 7]. CB1 is larger than CB2, with an additional 72 amino acid residues in the *N*-terminal region, 15 additional residues in the third extracellular loop, and 13 additional residues in the *C*-terminal region [12]. The highest degree of homology between CB1 and CB2 (44%) occurs in the transmembrane regions TM2, TM3, TM5, and TM6; interestingly, the homology in other regions is not particularly striking.

The CB1 cannabinoid receptors were found to be particularly enriched in cerebral cortex, hippocampus, basal ganglia, and cerebellum, regions that were predicted from the behavioural effects of CB. Lower levels were found in hypothalamus and spinal cord. Cannabinoid CB1 receptor mRNA is found primarily in neural tissue, but can be found to a lower extent in peripheral tissues, including the adrenal gland, bone marrow, heart, lung, prostate, testis. The CB2 receptor is found in immune tissues, such as the spleen, thymus and tonsils, and immunocompetent blood cells, with the highest concentration in B-cells, natural killer cells, mast cells, and monocytes ([1] for a rev.).

Both types of receptors belong to the superfamily of the "7 transmembrane-domain", and their functional response is mediated by pertussis toxin-sensitive GTP-binding ($G_{i/o}$) proteins. The signal transduction mechanism of CBs includes inhibition of AC, regulation of Ca^{2+} and K^+ channels (CB1 only), activation of MAPK, and other signal transduction pathways [10, 13–17]. Inhibition of AC and subsequent drop in cAMP decrease activation of cAMP-dependent protein kinase, PKA,

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Abbreviations used: AEA – anandamide, *N*-arachidonylethanolamide; AC – adenylate cyclase; CB (CBs) – cannabinoid(s); ERK – extracellular signal-regulated kinase; FAK – focal adhesion kinase; JNK – c-Jun N-terminal kinase; MAPK – mitogen-activated protein kinases; Met-AEA – *R*(+)-methanandamide; Met-F-AEA – 2-methyl-arachidonyl-2'-fluoro-ethylamide; NAE – *N*-acylethanolamines; NAPE – *N*-acylphosphatidylethanolamines; FAAH – fatty-acid amide hydrolase; 2-AG – 2-arachidonylglycerol; COX-2 – cyclooxygenase 2; OEA – *N*-oleoylethanolamine; PKC – protein kinase C; PLD – phospholipase D; PRL – prolactin; PEA – *N*-palmitoylethanolamine; ROS – reactive oxygen species; SEA – *N*-stearoylethanolamine; THC – Δ^9 -tetrahydrocannabinol; VEGF – vascular endothelial growth factor.

which leads to decreased phosphorylation of the K⁺ channels. In addition to inhibiting AC, CBs have been shown to stimulate cAMP accumulation possibly via activation of G_s [18].

1.2. Vanilloid receptors

The NAEs are also capable of activating another type of receptor — the vanilloid receptor [9, 19–23]. In particular, AEA is a full agonist for the receptors of another class of bioactive fatty acid amides, the *N*-acylvanyllamines (e.g. capsaicin and olvanil). These sites of action are known as vanilloid receptors of type 1 (TRPV1 — transient receptor potential vanilloid 1) [24].

Since CB1 stimulation may have opposite effects to those caused by TRPV1 stimulation, the presence of CB1 antagonists may enhance the apparent potency of anandamide at TRPV1, and this is important in tissues where the two receptors are co-expressed [20].

The transient receptor potential channel of melastatin type 8 (TRPM8), which is gated by low (< 25 °C) temperature and chemical compounds, is regulated by PKC-mediated phosphorylation in a way opposite to that observed with the TRPV1 [25].

The finding that the levels of AEA increase in inflamed ileum of rats treated with toxin A, and that subsequently AEA mediates the inflammatory effects of toxin A through a TRPV1-dependent mechanism, represents a typical example of how anandamide can activate TRPV1 under pathological conditions [26].

1.3. Non-CB receptor

However, both cannabinoids and endocannabinoids were found to affect various physiological functions by mechanisms, which are not dependent on receptor subtypes. Pharmacological and biochemical data suggest the existence of non-CB1, non-CB2 receptors activated *in vitro* by physiologically relevant concentrations of AEA [22, 27]. A novel, non-CB1 cannabinoid receptor has been proposed to mediate the inhibitory effect of cannabinoid agonist WIN 55212-2 on glutamate release in hippocampal pyramidal cells, and this putative receptor might bind also some vanilloid receptor ligands [27].

2. METABOLISM

In their fundamental review, Schmid and colleagues summarized data on *N*-acylethanolamine phospholipids (NAPEs) and NAEs biosynthesis and catabolism [28]. In mammals, NAPEs are formed by a membrane-associated *N*-acyltransferase that transfers the *sn*-1 acyl group of a donor phospholipid to the amino group of ethanolamine phospholipids [4, 28]. NAPE is only present in very small amounts in mammalian tissue. A significant increase in the formation of NAPE and NAE, including AEA, is seen when neurons are exposed to a neurotoxic environment [4, 28].

The formation of NAEs, including the CB receptor ligand AEA, is considered to be catalyzed by NAPE-hydrolyzing phospholipase D-type enzyme abbreviated to NAPE-PLD, which exhibits catalytic properties different from other PLD enzymes.

Some studies had focused on the proposed precursor for anandamide, *N*-arachidonoyl-phosphatidylethanolamine leading to: its quantitation in rat brain, characterization of PLD catalyzing its conversion to anandamide and the enzymatic transfer of an acyl group from the *sn*-1 position of phospholipids to the *N*-position of phosphatidylethanolamine, catalyzed by a Ca²⁺-dependent *N*-acyltransferase [29]. Further studies identified a calcium-independent PLD activity in brains from NAPE-PLD knockout mice that accepted multiple NAPEs as substrates, including the AEA precursor — NAPE (20 : 4) [30].

The effect of saturated and monounsaturated NAEs on phospholipase A2 (PLA2) activity is dependent on lipid bilayer features. The acyl chain length and the presence of a single double bond are crucial for the enzymatic activity modulation by NAEs. In fact, saturated NAEs with 10 carbon atoms do not affect the PLA2 activity, while NAEs with 12 and 16 carbon atoms largely activate the enzyme [31].

Activation of metabotropic receptors coupled to the phospholipase C (PLC) and diacylglycerol lipase pathway will systematically lead to increases in 2-AG production [16].

Alternative pathway for NAE biosynthesis proceeds through the serine hydrolase-catalyzed double-deacylation of NAPE to generate glycerophospho-NAE, followed by the phosphodiesterase-mediated cleavage of this intermediate to liberate NAE. A heretofore-uncharacterized enzyme alpha/beta-hydrolase 4 as a lysophospholipase/phospholipase B that selectively hydrolyzes NAPEs and lysoNAPEs, was isolated and identified [32].

Termination of the activity of the NAE is principally mediated by the integral membrane enzyme fatty acid amide hydrolase (FAAH) [8, 33, 34]. However, FAAH is an intracellular enzyme, so anandamide must be internalized to be hydrolyzed. Most evidence has pointed to a sodium- and energy-independent transporter operating by facilitated diffusion as the endocannabinoid uptake mechanism. FAAH has been identified as mostly responsible of AEA and, in some cases, 2-AG hydrolysis to arachidonic acid and ethanolamine or glycerol, respectively [35]. FAAH is a protein consisting of 597 amino acids with a high degree of conservation between mouse and human, and it has been cloned from a wide range of species. FAAH-like enzymes have been described in peripheral tissues such as ovaries, uterus, basophils [29]. In the latter cell type, FAAH, unlike the enzyme from brain and neuronal cells, displayed a high affinity toward PEA. The possibility of this being due to the existence of a FAAH isoform needs further investigation. Global metabolite profiling studies of FAAH(-/-) mice have recently identified a second class of endogenous FAAH substrates: the *N*-acyl taurines (NATs). To determine the metabolic and signaling functions performed by NAEs and NATs *in vivo*, a FAAH variant that discriminates between these two substrate classes would be of value [34]. 2-AG hydrolases, known as monoacylglycerol lipases

(MAGLs), and present in both membrane and cytosolic subcellular fractions, can catalyze 2-AG enzymatic hydrolysis. MAGLs recognize as substrates also other unsaturated monoacylglycerols, which in some cases compete with 2-AG inactivation [16].

Cyclooxygenase 2 (COX-2) is responsible for the metabolism of anandamide into prostaglandin-ethanolamides (PG-EAs) [36, 37].

Regarding the lipoxygenase products of anandamide and 2-AG, they can be formed through the action of 12- and 15-, but not 5-lipoxygenases. The 12-hydroxy-derivative of AEA still binds to CB receptors while the 15-hydroxy-derivative does not, but it inhibits FAAH. Unidentified hydroxy-derivatives of AEA have been suggested to act, like AEA, via vanilloid TRPV1 receptors. The 15-hydroxy-derivative of 2-AG was recently shown to be formed in eukaryotic cells, and its potential biological actions as a peroxisome proliferator-activated receptor α (PPAR α), but not PPAR γ , agonist was also investigated (rev. in [38]).

3. ANTINEOPLASTIC AND CYTOTOXIC EFFECTS OF CANNABINOIDS

3.1. Δ^9 -tetrahydrocannabinol and other exocannabinoids

Since first references on antineoplastic action of Δ^9 -tetrahydrocannabinol (THC) and other CBs on lung adenocarcinoma and L1210 leukemia cells [39, 40], there has been much evidence of cytotoxic, proapoptotic and antineoplastic effects of CBs *in vivo* and *in vitro*. Plant-derived CBs, such as THC, induce apoptosis in human Jurkat leukemia T cells [41], in dendritic cells [42], in macrophages and lymphocytes [43], in human prostate PC-3 cells [44], in transformed murine T cells [45], in splenocytes of C57BL/6 mice [46], in MiaPaCa2 and Panc1 human pancreatic tumour cell lines and in tumour xenografts obtained by s. c. or intrapancreatic injection of MiaPaCa2 cells [47], in EVSA-T, MDA-MB-231, MDA-MB-468, and SKBr3 human breast cancer cell lines [48], in leukemic cell lines CEM, HEL-92, HL60 and in peripheral blood mononuclear cells [49], in C6 glioma cell line [50–54], in primary neurons and hippocampal slices [55]. THC also inhibited glioblastoma tumour cell proliferation *in vitro* and *in vivo* [56]. Treatment of murine tumour cells EL-4, LSA, and P815 with THC *in vitro* results in a reduction in cell viability and an enhanced apoptosis [57]. Exposure of rat cortical neurons to THC at a concentration as low as 0.1 mM significantly decreased their survival, and at 5 mM THC after 2 h exposure doubled dead cells compared to a control culture [58]. These data were confirmed by the observation that THC induces morphological degenerative changes in cultured cortical neurons consistent with apoptosis [59]. The neurotoxicity of THC and synthetic CB — WIN 55,212-2, can be due to its ability to increase endogenous extracellular glutamate levels in primary cultures of rat cerebral cortex neurons [60]. The toxicity of THC was confirmed also in the *in vivo* experiments [61].

Cannabidiol, a nonpsychoactive CB of marijuana decreases the viability of U87 and U373 human glioma cell lines both *in vitro* and *in vivo* [62]. It caused apoptosis in more than 50% of U87 cell population and 40% of U373 cells. The CBs: THC, cannabidiol, cannabinol and cannabigerol inhibited proliferation of hyperproliferating human keratinocyte cell line [63]. Cannabidiol induced apoptosis in human leukemia cells [64] and inhibited the growth of xenograft tumours obtained by s.c. injection into athymic mice of human MDA-MB-231 breast carcinoma and rat *v-K-ras*-transformed thyroid epithelial cells [65].

3.2. Synthetic cannabinoids

Synthetic CB receptor agonists CP 55,940, WIN 55,212-2 and JWH-015 inhibited proliferation of rat C6 glioma cell [52] and human prostate cells [66]. Cannabinoids HU-210 and JWH-015 induced apoptosis in EL-4 tumour cells [57]. CB1/CB2 agonist WIN-55,212-2 induced apoptosis in androgen-responsive human prostate cells LNCaP [66, 67], in mantle cell lymphoma and malignant B-cell lymphoma [68, 69]. Selective CB2 agonist JWH-133 induced a regression of tumours xenograft of C6 glioma and human astrocytoma cells [54]. Agonists WIN 55,212-2, JWH-133, HU-210 caused apoptosis in nonmelanoma skin tumour *in vivo* and in tumourigenic epidermal cell lines PDV.C57 and HaCa4 *in vitro* [70]. WIN 55,212.2, HU-210, JWH-133 as well as THC induced apoptosis in the human umbilical vein endothelial cells thus inhibiting angiogenesis [71, 72].

3.3. Endocannabinoids

There is much evidence of cytotoxic and antineoplastic effects of endogenous CBs. The best studied endogenous cannabinoid, AEA, inhibited C6 glioma cell proliferation [52], dose-dependently inhibited the proliferation of MCF-7 and of human breast carcinoma EFM-19 cells [73, 74], caused apoptotic effects in human prostatic cancer cells [75], in pheochromocytoma PC12 cells [76–78], in Chang liver cells [79], in dendritic cells [42], in EL-4 murine tumour cells and human leukemia and lymphoma cell lines Jurkat, Molt-4 and Sup-T1 [57], in cultured rat cortical neurons and cerebellar granule cells [80], in the human umbilical vein endothelial cells [81], in MG63 osteosarcoma cells [82], in mantle cell lymphoma [68]. AEA induced caspase-independent necrosis-like cell death in primary hepatic stellate cells [83]. It is noteworthy that not only tumour cells but also primary hepatic stellate cells are highly sensitive to AEA-induced necrosis [83, 84]. AEA within the pathophysiologic range induced apoptosis of hepatoma cell line and primary hepatocytes [85]. AEA but not 2-AG amount increased during ischemia/reperfusion injury and enlarged infarct zone volume [86].

A metabolically stable analogue of anandamide, 2-methyl-arachidonyl-2'-fluoro-ethylamide (Met-F-AEA), inhibited C6 glioma cell proliferation [52], EFM-19 cell proliferation [74], induced growth inhibition and apoptosis in mantle cell lymphoma and malignant B-cell lymphoma [69], induced apoptosis

in H4 human neuroglioma cells [36, 87], suppresses Ras activity in *K-ras*-transformed FRTL-5 cells *in vitro* and FRTL-5 cell-derived tumours *in vivo* [88].

Endogenous CB1/CB2 receptor ligand 2-AG significantly inhibited rat C6 glioma cell proliferation, reduced the growth of colon cancer, invasion of prostate cancer PC-3 cells, inhibited the proliferation of PRL-responsive human breast cancer cells and prostate cancer DU-145 cells [52, 89–91].

Like AEA, other NAEs also induced apoptosis or necrosis in number of cell lines and tissues. Thus, *N*-stearoylethanolamine (SEA) was almost as effective as AEA in inducing apoptosis in rat glioma C6 cells [6, 92]. *N*-oleoylethanolamine (OEA) in rat glomerular mesangial cell reduced PDGF-induced proliferation by inhibiting sphingosine production [93] and enhanced TNF α -dependent ceramide accumulation followed by DNA fragmentation [94]. In embryonic neurons [95] and neurotumour cells [96] OEA has been reported to potentiate staurosporine-induced ceramide accumulation and apoptosis. OEA also enhanced ceramide-dependent apoptosis in B-lymphocyte cell line, WEHI 231 [97]. The proapoptotic effect of OEA can be explained by inhibition of the ceramidase, enzyme degrading ceramide, which is known as a potent proapoptotic agent [98].

A mixture of NAEs, containing saturated and unsaturated fatty acyls, enhanced apoptosis in human adrenal tumour tissue of adenocarcinoma, aldosteroma, androsteroma, hormonally inactive tumour, pheochromocytoma, fibroma and in adjacent to tumoral conventionally normal tissue of aldosteroma, androsteroma, hormonally inactive tumour and pheochromocytoma [99]. NAE also significantly enhanced proapoptotic effects of colchicine and cytochalasine B in aldosteroma and hormonally inactive tumour. SEA enhanced apoptotic processes in tumour tissue of corticosteroma. The action of high (micromolar) concentration of NAE on adrenal tumour tissue of aldosteroma caused necrosis-like DNA degradation (Figure) [100]. It is worth noting that labelled PEA incorporated mainly in adrenal tissue [101] that makes adrenal tumours suitable target for NAE treatment. SEA inhibited growth and decreased the volume of metastasis of Lewis carcinoma [102]. The effects of NAEs on the growth of various cancer cell lines *in vitro* and accumulation of NAE and their precursor phospholipids in various human tumours and some adjacent unaffected tissues was reviewed [103]. PEA and AEA quantity increase after focal cerebral ischemia and synergistically enhance microglial cell motility, and can thus participate in the propagation of focal cerebral ischemia-induced inflammation within the CNS [104].

NAEs can cooperate with AEA in apoptosis. OEA acts as an enhancer of AEA cytostatic effects on human breast carcinoma EFM-19 cells through competitive inhibition of its degradation [73]. PEA also enhanced the AEA-induced, CB1 receptor-mediated, antiproliferative effect in human breast cancer cells, possibly due to downregulation of the FAAH expression and positive modulation of VR1-mediated effects of AEA [24, 105].

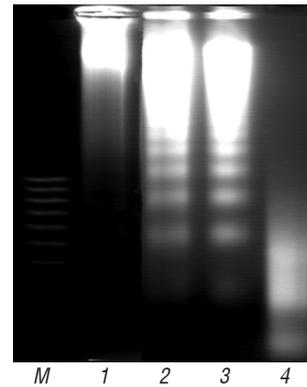


Figure. Effect of different NAE (18 : 0) concentrations on DNA fragmentation in aldosteroma tissue. M — marker (100–1000 b. p.), 1 — control, 2 — NAE (10^{-8} M), 3 — NAE (10^{-6} M), 4 — NAE (10^{-4} M)

NAEs are likely to exert destructive effects through their metabolites — ethanolamine, fatty acids and products of their further metabolism [33, 106, 107]. The toxicity of saturated fatty acids in different tissues and cells was well documented, although mechanism of induced apoptosis should be elucidated. Palmitate induces apoptosis via a direct effect on mitochondria with concomitant reduction of mitochondrial transmembrane potential followed by an increase in the production of ROS in 2B4.11 T cell hybridoma cell line and human hepatoblastoma cell line [108, 109]. Palmitate-induced apoptosis in neonatal rat cardiomyocytes was associated with a release of cytochrome C from mitochondria and a subsequent loss of mitochondrial membrane potential, however, no ROS involvement was observed [110]. It was shown that palmitate incorporates into ceramide and induces apoptotic DNA fragmentation in astrocytes [111]. The involvement of ceramide in apoptosis induced by saturated free fatty acids (palmitic and stearic) was shown in rat testicular Leydig cells [112]. On the other hand, apoptosis induced by saturated fatty acid appeared to be ceramide-independent in liver and Chinese hamster ovary cells [113, 114]. In CHO cells palmitate increased ceramide synthesis but palmitate-induced apoptosis occurs via generation of ROS [113]. In hepatic cells, fatty acids toxicity was mediated by TNF- α [115].

It is noteworthy that the amount of NAEs with most apoptogenic fatty acids is abundant in stressed tissue. Thus, the cortex and hippocampus of mice contained OEA (20%), PEA (53%) and SEA (14%) of total NAEs [116].

Another mechanism of destructive action of NAE upon membrane bilayer may involve activation of phospholipase A2 [30] with subsequent formation of lysophosphatidates toxic for cell.

3.4. Angiogenesis and metastasis inhibition

Cannabinoid receptor activation inhibits tumour growth, angiogenesis and metastasis formation [117]. Synthetic CBs inhibited angiogenesis in C6-cell gliomas, changing blood vessel morphology and inhibiting human umbilical vein endothelial cells migration. These processes were CB1/CB2-dependent and were attended by a decrease in the expression in the tumours of proangiogenic factors VEGF, angiopoietin-2 and matrix metalloproteinase-2 (MMP-2). The activity

of ERK is necessary for CB inhibition of cell migration [71]. WIN 55,212-2 and JWH015 inhibited the VEGF production in mouse gliomas and in cultured cells of rat C6 glioma, in human U373 MG astrocytoma, mouse PDV.C57 epidermal carcinoma, and human ECV304 bladder cancer epithelioma [72]. DNA array analysis showed that JWH-133 administration altered the expression of 10 genes related to the VEGF pathway, and among them CB treatment lowered the expression of VEGF-A, VEGF-B, the subunits of hypoxia-inducible factor-1 — HIF-1 α — the major transcription factor involved in VEGF gene expression [72]. Intratumoral administration of the THC to patients with glioblastoma multiforme decreased VEGF levels and VEGFR-2 activation in the tumours. The sphingolipid messenger ceramide is involved in the regulation of VEGF inhibition by CBs [72]. CB1/CB2 agonist WIN-55,212-2 and selective CB2 agonist JWH-133 induced growth inhibition of malignant epidermal xenograft tumours, increasing number of apoptotic cells. This was accompanied by an impairment of tumour vascularization, and decreased the expression of proangiogenic factors VEGF, placental growth factor, and angiopoietin-2. CBs also decreased the levels of EGF-R mRNA in tumours [70]. Met-F-AEA inhibited bFGF-stimulated endothelial cell proliferation, and induced apoptosis, via CB1 receptor [118]. The study of signaling pathways involved in angiogenesis showed that Met-F-AEA inhibited bFGF-induced ERK phosphorylation and activity of MMP-2. P38 MAPK evidently triggered proapoptotic pathways [118]. WIN-55, 212-2 treatment with human prostate cells LNCaP resulted in apoptosis induction and a decrease in VEGF [66]. In human breast cancer cells MDA-MB-231 and in murine breast cancer cell line TSA-E1 an Met-F-AEA inhibited migration of breast cancer cells and significantly reduced the number and dimension of metastatic nodes. CB decreased tyrosine phosphorylation of both FAK and Src. Authors supposed that inhibition of tumour cell invasion and metastasis occurred via modulating of FAK phosphorylation [119]. Cannabidiol reduced lung metastases deriving from intrapaw injection of MDA-MB-231 cells [65]. The inhibitory effect of AEA on both tumour SW 480 colon carcinoma cells and T-lymphocyte migration was reported. Specific inhibition of tumour cell migration via CB1 might be a selective tool to prevent metastasis formation without depreciatory effects on the immune system of cancer patients [120]. SEA decreased the volume of metastasis of Lewis carcinoma [102]. HU-331, quinone derivative of cannabidiol, effectively inhibited angiogenesis by inducing apoptosis in vascular endothelial cells [121].

Thus, antineoplastic properties of CBs make them promising agents for tumour therapy [2, 122]. Phase I trial in which nine patients with recurrent glioblastoma multiforme were administered with THC intratumorally, showed positive results [56]. There are also evidences that the endocannabinoid AEA and different synthetic CBs inhibit P-gp activity *in vitro* via a CB receptor-independent mechanism [123] that make them suitable for use in combination with other antineoplastic agents.

4. MECHANISMS OF CANNABINOID-INDUCED APOPTOSIS

4.1. Involvement of receptors

There are a number of evidences in favour of CB and other receptors mediating the apoptotic processes. CB receptor activation induces apoptosis of skin tumour cell [70]. Activation of CB1/CB2 receptors decreased growth, proliferation, angiogenesis and metastasis, and increased apoptosis of melanomas in mice [124]. Blockade of CB1 receptor decreased NMDA-induced excitotoxicity in the ipsilateral forebrain by reducing the infarction area and the number of cortical degenerating neurons [125].

The role of CB receptors in THC-induced cell death is still controversial. Early reports pointed to an unidentified CB receptor-independent mechanism [44, 53]; further investigations have shown that both CB1 and CB2 receptors can contribute to cytotoxic effect of THC [50] although in an unclear manner. In cortical neurons, THC triggered cell death mainly through a receptor-mediated mechanism [59]. In dendritic cells, the involvement of CB1 and CB2 receptors in THC-induced apoptosis has been proved. THC binds to both CB1 and CB2 with similar affinities. Gi/Go proteins are partially involved in the induction of apoptosis by THC [42]. The participation of CB1 and CB2 receptors and G proteins in THC-induced apoptosis was shown on Jurkat leukemic cells [41, 126], in culturing thymocytes [57], in met-AEA- and Win 55,212-2-induced apoptosis in mantle cell lymphoma and B-cell lymphoma [69]. THC and WIN-55,212.2, HU-210, JWH-133 induced apoptosis in the human umbilical vein endothelial cells via CB2 or both CB1/CB2 receptors [71, 72]. Synthetic CB receptor agonist WIN-55,212-2 acts through activation of both CB1 and CB2 receptors in human prostate cells [66]. JWH-133 mediates its effects in rat glioma C6 cell line via CB2 but not the CB1 [54]. The antiproliferative effect of cannabidiol was also dependent on CB2 receptor activation in culturing splenocytes [57] and in U87 and U373 human glioma cell lines [62]. CB1, VR1 vanilloid receptors and Gi/Go proteins were not involved in glioma cells cannabidiol-induced apoptosis [62].

Proapoptotic effects of the AEA and 2-AG upon C6 cells are realized via a mechanism involving combined activation of both vanilloid and to lesser extent CB receptors [52]. In human endothelial cells, AEA also induces apoptosis via VR1 [81]. In human neuroblastoma CHP100 and lymphoma U937 cells, AEA triggered apoptosis via vanilloid receptors whereas CB-receptor-signaling caused protective effect against apoptosis [127]. AEA-enhanced ischemia injury and infarction volume was mediated by CB1 receptors [86].

The endogenous C18 *N*-acylethanolamines — *N*-linolenylethanolamine (18 : 3), *N*-linoleylethanolamine (18 : 2), OEA (18 : 1), but not SEA (18 : 0) activated the native vanilloid receptors (TRPV1) in rodent blood vessels and the cloned hTRPV1 expressed in HEK293 cells [21] that suggest proinflammatory action of these NAEs. SEA binds to special sites on rat C6 glioma cells distinct from CB or vanilloid receptors

but an antagonist of vanilloid receptors partly inhibited SEA binding [92].

In contrast to these findings, CB receptors (CB1, CB2) and vanilloid receptor VR1 apparently were not involved in met-AEA-induced apoptosis in H4 human neuroglioma cells [36], and AEA-induced necrosis in primary hepatic stellate cells [83], in leukemic cell lines CEM, HEL-92, HL60 [49], in cultured rat cortical neurons and cerebellar granule cells [80], in hepatoma cell line and primary hepatocytes [85], in PC12, HL60, C6, Neuro-2a, CHO, HEK, SMC and Jurkat cells [128]. Effects of NAEs containing saturated fatty acyls also can be mediated via non-receptor mechanisms [129, 130].

Thus, the role of receptors in the triggering of apoptosis is not unequivocally definite. Evidently, participation of receptors in CB effects depends on tumour type, cell line, and tissue studied.

4.1.1. Lipid rafts

Perhaps lipid rafts can play a significant role in endocannabinoid- and especially NAE-induced apoptosis. In rat glioma C6 cells a raft disruptor - methyl- β -cyclodextrin (MCD) — reduces AEA-induced apoptosis [131]. Lipid rafts evidently control CB1 receptors, but not CB2 receptors, in immune and neuronal cells [7]. In primary hepatic stellate cells, MCD inhibited AEA binding, blocked ROS formation and intracellular Ca^{2+} increase, thus preventing necrosis [83]. Lipid rafts are involved also in AEA-induced apoptosis in Chang liver cells [79]. The AEA-induced cell death in PC12, C6, Neuro-2a, CHO, HEK, SMC, Jurkat and HL-60 cells was independent on CB1/CB2 or VR1 activation, but was completely blocked by MCD, thus indicating lipid rafts involvement in this process. MCD also blocked AEA-induced ROS generation and p38 MAPK/JNK activation [128]. Furthermore, cholesterol, present in the cell membrane and especially in lipid rafts plays an important role in AEA-induced apoptosis of hepatocytes, possibly functioning as AEA receptor [85].

The role of lipid rafts in endocannabinoid signaling and apoptosis triggering was reviewed and analyzed recently [132, 133].

4.2. Intracellular signaling

MAPK-dependent signaling obviously plays the essential role in the triggering of apoptosis by CBs. AEA treatment resulted in the activation of p38 MAPK, JNK, and p44/42 ERK1/2 in PC-12 cells [77], but only p38 and JNK are involved in the apoptosis, as well as upstream kinase - ASK1. The activation of ASK1, p38 MAPK, and JNK is accompanied by cytochrome C release from the mitochondria and caspase activation, suggesting that AEA triggers a mitochondrial-dependent apoptotic pathway. AEA initiated phosphorylation of JNK and p38 MAPK in human umbilical vein endothelial cells [81]. AEA-induced apoptosis in Chang liver cells involved activation of p38 MAPK and JNK pathway, with subsequent increase in c-Jun, JunB levels and transcriptional complex AP-1 DNA-binding activity [79]. The AP-1 transcriptional targets, FasL and Bim, also increased during AEA treatment, accompanied by an increase of the Bax and truncated

Bid levels, activation of caspase-3 and caspase-6 [79]. In hepatoma cell line and primary hepatocytes, AEA-induced cell death was preceded by G0/G1 cell-cycle arrest, activation of p38 MAPK and JNK, and inhibition of Akt-dependent survival pathways [85]. In human osteosarcoma MG63 cells, AEA triggered apoptosis via Ca^{2+} -enhanced Ca^{2+} influx and Ca^{2+} release with concomitant p38 MAPK activation. Phosphorylation of p38 MAPK subsequently activated caspase-3. Although AEA induced expression of ERK, JNK and p38 MAPK, first two kinases apparently were not involved in the apoptosis [82]. The same results were obtained on human leukaemia cells, where CB2 receptor stimulation induced caspase activation and apoptosis via p38 MAPK activation [134], and in lymphoma cell where met-AEA and Win 55,212-2 induced growth inhibition and apoptosis through phosphorylation of p38 MAPK [69]. In H4 human neuroglioma cells met-AEA caused a biphasic activation of the p38 MAPK and p42/44 ERK1/2 followed by ceramide synthesis [135].

AEA also inhibited IKKs, with subsequent inhibition of NF- κ B binding to DNA, and NF- κ B-dependent transcription in A549 lung adenocarcinoma cells [136]. The IKK and its downstream transcription factor NF- κ B are the key regulators of genes involved in the inflammatory response and in survival from apoptosis [137].

THC induces apoptosis in leukemic cells by suppression of the Raf-1/MEK/ERK/p90RSK survival pathway that causes partial dephosphorylation and translocation of proapoptotic Bad to mitochondria [41]. THC also decreased the phosphorylation of Akt with no effects on apoptosis. No changes in phosphorylation of p38 MAPK and JNK were observed. ERK inactivation was also showed in other human tumour cell lines - Molt4, Hut78, and SupT1 [41]. On the other hand, ERK activation mediated THC-, WIN-55,212.2-, HU-210- and JWH-133-induced apoptosis as well as inhibition of cell migration of the human umbilical vein endothelial cells. No effects of protein kinase A, protein kinase C or the p38 MAPK were observed [71]. Although the role of PKA in antiproliferative CB signaling is undeciphered, inhibition of PKA activity by 2-AG may be one of the inhibition effects on androgen-independent prostate cancer cell invasion [91]. In cortical neurons, THC induces apoptosis through CB1 receptors and JNK activation [58, 138]. It is possible that this process involves p53 suppressor protein [139].

In dendritic cells, THC initiated apoptosis using both death receptor and mitochondrial pathways. THC treatment induced cleavage of caspase-8, -9, and -2, cleavage of Bid and cytochrome C release [42]. The ERK1/2, p38 MAPK, JNK, and PI3K pathways apparently were not involved in THC-induced apoptosis as opposed to NF- κ B. cDNA microarray analysis showed that several apoptotic genes were significantly up-regulated in THC-treated dendritic cells when compared with control cells, and most of these genes showed NF- κ B binding site in their promoter, suggesting that NF- κ B may act as a proapoptotic molecule in THC-induced apoptosis [42]. In leukemic cell lines,

THC induced expression of genes in the Ras/MAPK pathway but did not increase the p53 expression [49]. There were no significant changes seen in genes involved in ceramide metabolism. THC caused ERK inactivation by dephosphorylation perhaps because of overexpression of *DUSP6* gene, encoding dual specificity phosphatase-6/MAPK phosphatase-3 and a decrease in MEK2 expression [49]. On the contrary, synthetic cannabinoid JWH-133 induces apoptosis via ERK activation and ceramide synthesis *de novo* [54]. Experiments with two subclones of C6 glioma cells in culture showed that CBs signal apoptosis by a pathway involving CB receptors, sustained ceramide accumulation and Raf1/ERK activation [50]. These data are confirmed by observation of sustained up-regulation of ERK1/2 and inhibition of PI3K/Akt pathways in WIN-55,212-2-treated human prostate cancer cells, both androgen-responsive LNCaP and androgen-independent PC3 [67]. WIN-55,212-2 activated in these cells the classical apoptotic pathway through Bax up-regulation, a decrease in Bcl-2 expression, increase in AIF, caspase-3 activation, and PARP cleavage [67]. ERK activation mediates prominent effect on cell cycle regulatory machinery: an induction of p53 and CDK inhibitor p27/KIP1; down-regulation of cyclins D1, D2, E; decrease in the expression of CDKs 4/6, and CDK2; decrease in protein expression of pRb; down-regulation of E2F(1–4); decrease in the protein expression of DP1 and DP2 and, as a result, an arrest of the cells in the G0/G1 phase of the cell cycle [67]. Antitumour effect of Met-F-AEA was associated with a strong decrease in Ras activity in thyroid epithelioma [88], which suggests inhibition of proliferative pathways. The effect of Met-F-AEA on thyroid tumour cells FRTL-5 was due to the arrest of the cell cycle at the G0/G1 phase, associated with a significant reduction the quantity of cells in the S phase [88]. In human breast cell, THC decreased the total levels of Cdc2 and enhanced levels of p21WAF, a CDK inhibitor causing G2/M arrest, and cell cycle arrest was associated with apoptosis. At a lower concentration, THC increased the number of cells in the G0/G1 compartment and, in parallel, decreased the number of cells in S phase [48]. CBs proapoptotic and antiproliferative action on melanoma cells was due, at least in part, to cell cycle arrest at the G1/S transition via inhibition of the Akt and dephosphorylation of the pRb [124]. The antiproliferative effect of the AEA on human breast cancer cells, MDA-MB-231 is also mediated by cell cycle arrest. Met-F-AEA, induces an S phase arrest correlated with Chk1 activation, Cdc25A degradation, and Cdk2 activity suppression [140].

4.2.1. Ca²⁺

Ca²⁺-signaling evidently plays a significant role in CB effects. The ability of CBs, and AEA primarily, to modulate the P-type high-voltage-activated calcium currents and other types of Ca²⁺ channels, such as voltage-dependent Ca²⁺ channels (VDCCs), voltage-gated L, N, and P/Q Ca²⁺-currents [1, 141], is well known.

AEA increased Ca²⁺ levels and caused Ca²⁺-dependent cell death in canine kidney cells [142], in human neuroblastoma CHP100 and lymphoma U937 cells [127]. Ca²⁺ increase depends on both extracellular ion concentration, and intracellular store mobilization. PKC and AC may play negative role in Ca²⁺-mediated cell death [142]. In both rat cortical neuronal and cerebellar granule cells apoptosis was associated with changes in mitochondrial membrane potential and increased levels of intracellular Ca²⁺ [80]. SEA exerts proapoptotic effects on rat C6 glioma cells through an increase in intracellular calcium, activation of the arachidonate cascade, and mitochondrial uncoupling [92].

Interestingly, in human embryonic kidney 293 cells and in cultured hippocampal neurons only WIN55,212-2, but not THC, HU-210, CP55,940, 2-AG, met-AEA, and cannabidiol, increased intracellular calcium. This increase was independent on G_{i/o}, and was mediated by G_q G proteins and phospholipase C [143]. This is another evidence that in different cell types CB effects can use different signaling mechanisms.

4.2.2. Ceramide

Ceramide is a membrane sphingolipid that can be generated by ceramide synthase, a key enzyme involved in *de novo* sphingolipid biosynthesis or, alternatively, ceramide can be formed as a result of sphingomyelin hydrolysis by sphingomyelinases.

Recent investigations indicate that several actions of CBs, such as inhibition of cell growth, effects on energy metabolism, stimulation of ketogenesis and glucose metabolism, are mediated through accumulation of the lipid second messenger ceramide [144] for rev.

Structurally the ceramide and NAE molecules are close enough [145], and OEA is known as a potent inhibitor of the ceramidase [98] — an enzyme that metabolizes ceramide, involved in the regulation of apoptosis [146].

As mentioned above [50, 54, 135], ceramide is implicated in proapoptotic action of CBs. Incubation of MiaPaCa2 and Panc1 human pancreatic tumour cell lines with THC led to sustained ceramide accumulation, indicating that synthesized ceramide is involved in THC-induced apoptosis of pancreatic tumour cells [47]. *De novo* synthesized ceramide has been implicated in CB receptor-mediated apoptosis induced by THC in glioma cells [50, 52, 54, 144, 147]. In C6.9 glioma cells, THC stimulated sphingomyelin hydrolysis. THC and *N*-acetylsphingosine — a cell-permeable ceramide analogue — induced apoptosis in several transformed neural cells but not in primary astrocytes or neurons [53]. CB2 receptor-dependent stimulation of ceramide biosynthesis was necessary for caspase activation and apoptosis in human leukemia Jurkat cells [126]. The apoptosis was developed via mitochondrial pathway with cytochrome C release and loss of mitochondrial membrane transmembrane potential, although caspase-8 activation was also observed.

Ceramide may be involved in AEA-induced apoptosis because the selective inhibitor of its biosynthesis fumonisin B1 significantly inhibited the endocan-

nabinoid effect upon C6 cells [52]. AEA effects were also mediated by production of ceramide in Chang liver cells [79]. In mantle cell lymphoma and B-cell lymphoma, met-AEA and Win 55,212-2 induced apoptosis via consequent accumulation of ceramide, phosphorylation of p38 MAPK, depolarization of the mitochondrial membrane, and caspase activation [69]. In H4 human neuroglioma cells met-AEA increased in intracellular ceramide level [135]. The activation of the p38 MAPK and ERK1/2 was necessary for met-AEA effects [135]. OEA enhanced accumulation of long-chain ceramide and potentiated the apoptosis induced by C6-ceramide [148]. Addition of OEA to cell cultures increased the endogenous level of ceramide and stimulated apoptotic processes [5, 94–97, 149].

The involvement of ceramide in the process of VEGF inhibition by WIN-55,212-2 in cultured glioma cells and in mouse gliomas was shown [72].

Recent studies also demonstrated that ceramide directly participates in the regulation of cardiac function. The sphingomyelin-ceramide signaling pathway was activated by myocardial ischemia/reperfusion *in vivo*, and hypoxia/reoxygenation *in vitro* activated sphingomyelinase to produce ceramide (for rev. see [150]). Exogenous cell-permeable ceramide has been shown to induce cardiomyocyte apoptosis *in vitro*, which contributes to myocardial ischemia/reperfusion injury. In addition, the ceramide metabolite sphingosine in adult mammalian cardiac myocytes inhibited the intracellular Ca^{2+} mobilization, facilitating the development of ischemia/reperfusion injury. Ceramide-mediated signaling is a novel mechanism underlying endothelial dysfunction associated with overproduction of cytokines during ischemic heart disease [150].

On the other hand, inhibitors of serine-palmitoyl-transferase and ceramide synthase, as well as inhibitors of acid and neutral sphingomyelinase, had no effect on the cellular effects of cannabidiol in U87 glioma cells [62]. There was no involvement of ceramide in cannabidiol-induced apoptosis, thus suggesting that the cannabidiol mechanism is clearly different from that described for THC and AEA [52].

Overall, data analysis shows that CB receptors are coupled to the generation of the lipid second messenger ceramide via two possible pathways: sphingomyelin hydrolysis and ceramide synthesis *de novo*. Sustained ceramide accumulation in tumour cells mediates CB-induced apoptosis, as evidenced by *in vitro* and *in vivo* studies [122, 151]. This effect in glioma cells seems to be due to the ceramide activation of the ERK cascade and the inhibition of Akt pathway [3, 122, 151].

4.2.3. COX-2

Besides a stimulatory action on the expression of a variety of proinflammatory mediators, ceramide has been reported to induce COX-2 expression — another possible mechanism of apoptosis induction. It involves activation of COX-2, but not COX-1, and prostaglandin E_2 synthesis. Met-AEA enhances COX-2 expression in human neuroglioma cells [36, 37, 135, 152] with following prostaglandin E_2 synthesis [135]. Met-AEA-induced

COX-2 expression in neuroglioma cells was mediated by ceramide synthesis and subsequent activation of p38 and p42/44 MAPK pathways [135]. AEA inhibited the growth and induced cell death (neither apoptosis nor necrosis) of colorectal carcinoma cell lines with high (HT29) and moderate (HCA7/C29) COX-2 expression, but had little effect on the low COX-2 expressing cell line SW480. AEA-induced cell death was partially COX-2-dependent, mediated via metabolism of anandamide by COX-2, rather than its degradation into arachidonic acid and ethanolamine. It is interesting that prostaglandin-ethanolamides PGE₂-EA and PGD₂-EA induced classical apoptosis [153]. Hippocampal neuronal death caused by THC also was associated with an increased production of PGs. [55].

4.2.4. Oxidative stress

AEA-induced apoptosis in Chang liver cells involved oxidative stress [79]. Sarker et al. showed that AEA-induced increase in intracellular superoxide levels caused apoptosis in PC-12 pheochromocytoma cells [76]. AEA raised intracellular superoxide level in C6, Neuro-2a, CHO, HEK, SMC, Jurkat and HL-60 cells [128], increased susceptibility to oxidative stress-induced hepatocyte damage [85]. Antiproliferative effect of AEA and 2-AG was completely blocked by incubation of the C6 glioma cells with the antioxidant α -tocopherol, and greatly reduced with the cell-permeable calpain inhibitor calpeptin. These data pointed out the important role of calpain activation and, especially, oxidative stress in the antiproliferative action of CBs [52]. Oxidative stress may play key role in the glioma cell death. Massi et al. hypothesized that the effect of cannabidiol could be attributed to ROS production that can mediate the cell death in human glioma cells [62]. This assumption was confirmed by the finding that in primary hepatic stellate cells AEA induced necrosis via ROS formation, and an increase in intracellular Ca^{2+} [83]. SEA inhibited growth and quantity of metastasis of the Lewis carcinoma perhaps due to decreasing the antioxidant activity [102]. Monounsaturated OEA enhanced fatty acid oxidation in skeletal muscle strips, dissociated hepatocytes, and primary cardiomyocyte cultures by activating the nuclear receptor PPAR- α [154].

The cytotoxic effects of NAE can be the result of the lipoxygenase activity that catalyzes the conversion of polyunsaturated fatty acids into conjugated hydroperoxides with proapoptotic properties [106]. NAE metabolites — saturated fatty acids and products of their oxidation — can cause destructive action through ROS generation [108, 109, 113].

Ceramide induced apoptosis of human papilloma virus cervical cancer cells, which was associated with an increase in ROS and nitric oxide production, a loss in mitochondrial membrane potential, an increase in NF κ B translocation, and a decrease in reduced glutathione concentration [155].

Other experiments indicate on both antioxidative and prooxidative effects of PEA. The antioxidative effect is obtained at low PEA concentrations (0.01 and

0.1 μM), while the prooxidative effect is obtained at a higher PEA concentration (1 μM) [156]. The elevation in the levels of ROS during low serum-induced apoptosis is not affected by AEA [157]. AEA and OEA can even decrease ROS formation through inhibitory effects of these NAEs on the respiratory chain in rat heart mitochondria [158].

4.2.5. Other signaling mechanisms

AEA and other CBs induce NO generation in different tissues. AEA effects on endothelial cells may lead to vasodilatation through a CB1-mediated increase in NO [159]. AEA and HU-210 stimulated NO production in cultured human umbilical vein endothelial cells [127]. AEA and 2-AG stimulated the nitric oxide synthase (NOS) activity [141]. AEA and met-AEA induce acute release of NO that was paralleled by a 2-fold increase in cGMP tissue levels [23]. AM404, an inhibitor of AEA uptake, completely prevented the overproduction of NO and the overexpression of nNOS, inhibited the increase in TNF α and in the ratio bax/bcl-2 expression [160]. Although there are no direct connections between CB-induced NO generation and apoptotic or necrotic processes, involvement of NO in apoptosis was well documented [155, 161, 162], and it is possible to suppose the involvement of NO (NOS) in AEA-triggered apoptosis because NO-induced PC-12 cell death is attended by p38 MAPK activation [77].

In breast cancer cells met-F-AEA decreased tyrosine phosphorylation of both FAK and Src [119]. Earlier the activation of FAK by AEA and 2-AG was noted [1, 141], indicating involvement of cytoskeleton in signal transduction.

A stress-regulated protein p8 related to the architectural factor HMG-I/Y is involved in THC-induced apoptosis of pancreatic tumour cells. Its levels increased after THC treatment of MiaPaCa2 cells, and several p8-dependent genes have been implicated in apoptotic signaling. Among these genes, the endoplasmic reticulum stress-related proteins ATF-4 and TRB3 are suggested as potential mediators of p8-dependent effects in these cells [47, 163].

Thus, CB-induced cell death can develop mainly via receptor-dependent pathways and involve MAPK activation (p38 MAPK in the first place), Ca²⁺ increase, ceramide production, ROS formation, PI3K/Akt survival pathways inhibition and cell cycle impairment.

5. PROTECTIVE EFFECTS OF CANNABINOIDS

Numerous evidences on protective effects of exocannabinoids and synthetic CBs on different tissues were obtained [147, 164–174]. There are less data on protective effects of AEA [157, 175–178], other NAE and their precursors [165, 179–183].

The contradictory data on the pro- and antiapoptotic effects of CBs are difficult to explain. The end result may depend on experimental models and settings used. As was pointed out by Mendizabal and Adler-Graschinsky, studies performed *in vivo* have shown discrepancies according to the state of the experimental animals (an-

aesthetized or conscious), the route of administration (central or peripheral), and the doses of the compound used. Moreover, the effects of CBs may be influenced by either the nature of the experiment (*in vitro* or *in vivo*) or the type of *in vitro* preparation (isolated vessel or perfused vascular bed) [184].

Guzman [3] stated that CBs protect normal neurons and glial cells from apoptosis induced by toxic insults, such as glutamatergic overstimulation, ischemia and oxidative damage, and enhance apoptosis in tumour cells. It suggests that CBs regulate cell survival and cell death pathways differently in tumour and non-tumour cells [3]. It may be true for glioblastoma cells — the best studied experimental model. This statement was confirmed by the observation that human breast cancer cells with more aggressive phenotype were more sensitive to THC, whereas human non-tumour mammary epithelial cells were more resistant to CB treatment [48], indicating a certain degree of selectivity of cannaboids action. However, there are abundant data that evidence a cytotoxic, proapoptotic action of CBs on nontumoral — normal or conventionally normal cells and tissues [42, 43, 46, 49, 55, 59, 63, 71, 80, 81, 83, 85, 99, 118, 121] that raised doubts in such suggestion.

6. NAE: PROTECTORS OR KILLERS?

The situation with NAE effects on different tissues is of special interest.

N-acylethanolamine lipids NAE and NAPE have been detected in trace amounts in various mammalian tissues [28]. The levels of these compounds markedly increase during the degeneration of tissues or cells under stress conditions. Thus, AEA, other NAEs and their precursors, NAPEs, accumulate during brain injury and postmortem [185–188]. Levels of various NAEs including AEA were dramatically increased in CdCl₂-administered rat testis. *N*-palmitoyl species increased 39-fold, *N*-stearoyl species — 21-fold, and AEA — 5-fold. Also, there was an increase in several species of NAPE, precursors for NAEs [189]. *N*-acyl lipids accumulate in cultured neocortical neurons subjected to sodium azide-induced cell injury. Exposure to sodium azide increased the total amount of NAPE three-fold over control levels [190]. NAE and NAPE content also increase in myocardial infarction [191], glutamate-induced neuronal cytotoxicity [192], UV light irradiation of epidermal cells [193], and in yeast exposed to oxidative stress [194]. Elevated levels of AEA were associated with both apoptotic and necrotic neuronal cell death [195]. AEA levels were upregulated in the hippocampus following glutamate excitotoxicity [196], in models of Alzheimer's and Parkinson's disease [197] and in focal cerebral ischemia [86].

The dramatic increase in NAE amounts under stress conditions led Schmid et al. to the assumption that these compounds could play a protective role in degenerating tissues [28]. At that time there were too little evidence supporting such hypothesis but even now Matas et al., which showed protective effect of AEA in neuroblastoma cells, concluded after all that it is not

clear if the increase in AEA levels under stress conditions could point to a protective effect of AEA or might be part of the mechanism causing the pathological manifestation and/or its consequences [157]. Indeed, NAE accumulation under conditions of tissue damage itself cannot be evidence of their protective action. It is important to note that NAPEs and NAEs accumulate in the brain in response to neurodegenerative insults at a time when other phospholipids are subjected to rapid degradation [5], which can lead finally to disintegration and digestion of cell membranes.

The first in line of evidences on possible protective effects of NAE was the finding that NAE at high concentrations inhibited non-specific Ca^{2+} permeability increase of mitochondrial membrane induced by oxalacetate [179]. However, the same group demonstrated that NAE dramatically increased the permeability of membrane of sarcoplasmic reticulum to Ca^{2+} [198], which may have negative consequence in ischemic myocardium.

NAE partly normalized the phospholipid content and decreased Rb^{+} -efflux in veratridine-treated neuroblastoma cells [180]. These effects of NAE actually cannot be considered as protective because cell viability was not determined in this experiment. Besides, NAE *per se* significantly increased Rb^{+} -influx in controls, which cannot be attributed to its stabilizing effects upon membranes [180]. On the other hand, NAE cause haemolysis in trout red blood cells and its haemolytic effect was proportional to chain length [199]. The influence of *N*-acyl chain length could be related to disordering effects of *N*-acyl methyl groups on the hydrocarbon chains packing.

NAPEs with saturated *N*-acyl groups in *in vitro* experiments have been found to form lamellar structures that have given rise for assumption on their membrane-stabilizing effects [5, 200]. However, whether such a membrane-stabilizing effect can be realized *in vivo* is unknown.

Next, Parinandi and Schmid showed that *N*-oleoyl-2-aminoethanol at high concentrations (50–150 mkM) inhibited the production of thiobarbituric acid-reactive substances in rat heart mitochondria [183]. *N*-Oleoyl-2-aminoethanol minimally affects peroxidation of linoleic acid micelles and has no effect on deoxyribose peroxidation. Some saturated NAEs (100 mkM/l) can affect the Fe^{2+} -induced free radical oxidation of lipids *in vitro* in liver mitochondria of rats with acute hypoxic hypoxia, although authors did not observe any statistically significant effects of PEA and SEA in mitochondria of intact rats [182]. Experiments on rat heart mitochondria showed that NAEs are mitochondrial uncouplers and openers of the permeability transition pore [201] that could promote apoptotic processes *in vivo*. Depending on experimental conditions, NAEs can enhance or inhibit the ROS production [158]. The results on isolated mitochondria differ from observations on intact cells. Thus, it was shown that AEA (10 mkM) initiated the generation of intracellular superoxide anion, which triggers downstream signals resulting in caspase-3 activation and apoptosis of

PC-12 cells [202] and in primary hepatic stellate cells [83]. The ROS-generating and oxidative effects of NAE significantly prevailed (see chapter 4.2.4 of this paper) its antioxidant, protective effects.

In conventionally normal tissue of human adrenals SEA attenuated the suppressive effect of mitotane on K^{+} -dependent steroidogenesis [203] that may be regarded as a protective action, although NAE exert proapoptotic effects on the same tissue [99].

The direct evidence of a protective effect of PEA at high (100 mkM) concentration against glutamate toxicity was obtained *in vitro* on cultured mouse cerebellar granule cells [165]. It is noteworthy that AEA antagonized these neuroprotective effects. The last fact is odd enough because glutamatergic insult causes a 13-fold increase of AEA *in vivo* [195]. It is also well documented that activation of CB1 receptors (AEA is a partial agonist of these receptors) is neuroprotective, most likely via a dominant presynaptic inhibition of glutamate release [125]. Thus, it is possible that extremely high concentrations of NAE and glutamate (500 mkM) used in experiments *in vitro* [165], could produce some non-specific effect. It should be noted that maximal amount of NAE in tissue under stress conditions did not exceed several mkM [28, 195].

The excellent analysis of protective properties of NAPE and NAE made by Hansen and colleagues [4, 5] showed that protective effect of NAPE *in vivo* may be not taken into account and that available limited data on protective action of NAEs were obtained mainly *in vitro*. They consider, however, the proapoptotic action of NAE as protective with regard to damaged tissue as opposed to necrosis that caused inflammation. It difficult to interpret the apoptosis as protective mechanism in general and besides NAEs can initiate necrosis as well, see Figure [83, 100].

CONCLUSION

I. Cannabinoids are promising agents both solo and in combination with other cancerostatics for preclinical investigation as potential anticancer therapeutic means.

II. The above-mentioned data led us to the hypothesis that NAEs and products of its further degradation are not protectors of the damaged tissue but are instruments of its death execution. That is to say, NAE formation at a strong stress conditions may be not a consequence of tissue damage but cause such damage.

The following evidences are in favour of such hypothesis:

1. Accumulation of NAEs with most apoptogenic acyl residues under different stress condition as a result of NAPEs degradation.
2. Triggering of apoptosis or necrosis in numerous tissues and cells, as well as enlarging the infarction area by NAE.
3. Toxicity of products of NAE degradation (saturated fatty acids in the first place).
4. Enhancing by NAE of ROS production (superoxide and NO).
5. Ceramide, mediating cytotoxic NAE effects, can *per se* cause myocardial infarction and stroke.
6. It has been shown that NAE are agonists of vanilloid receptors that in turn assume

its proinflammation action. 7. Actually, absence of evidences of protective effects of NAPEs and NAEs *in vivo*. Some supposed protective effects of NAE in high concentrations, obtained on very special *in vitro* systems (lipid vesicles or isolated mitochondria), may be due to physical lipid interactions that are not related to complex biological systems.

The NAE generation in degenerating tissue may be the first step of cellular membranes degradation at all. Perhaps at weak stress stimuli, when NAE quantity is relatively small, NAE could cause some protective, antioxidant effect. However, under strong stress conditions (hypoxia), NAE, produced in large amounts together with products of their further degradation, may represent a proapoptotic cascade that causes, depending on cell context, apoptosis or necrosis.

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АНТИНЕОПЛАСТИЧЕСКОЕ И АПОПТОТИЧЕСКОЕ ДЕЙСТВИЕ КАННАБИНОИДОВ. *N*-АЦИЛЭТАНОЛАМИНЫ: ЗАЩИТНИКИ ИЛИ УБИЙЦЫ?

В обзоре проанализированы апоптотические и антинеопластические свойства каннабиноидов с акцентом на эффекты *N*-ацилэтаноломинов. Каннабиноиды усиливают апоптотические и некротические процессы во многих типах опухолевых клеток и тканей. Обсуждается участие рецепторов разных типов и внутриклеточных сигнальных механизмов в опосредовании проапоптотических эффектов каннабиноидов. Оцениваются доказательства в пользу проапоптотических, пронекротических и защитных, антиапоптотических эффектов каннабиноидов и, особенно, *N*-ацилэтаноломинов. Выдвигается гипотеза, по которой *N*-ацилэтаноломины, которые образуются в некоторых тканях в условиях сильного стресса, могут быть не следствием повреждения ткани, а причиной и инструментом такого нарушения. Сделан вывод о перспективности использования каннабиноидов в качестве потенциальных противоопухолевых препаратов.

Ключевые слова: каннабиноиды, *N*-ацилэтаноломины, апоптоз, некроз, опухоль, ишемия.